39

Photobleaching

Alberto Diaspro, Giuseppe Chirico, Cesare Usai, Paola Ramoino, and Jurek Dobrucki

INTRODUCTION

Thanks to the wide variety of applications, fluorescence microscopy is now one of the most popular imaging techniques in biology (Weber, 1960; Lakowicz, 1999; Periasamy, 2001; Michalet *et al.*, 2003; Tsien, 2003; Bastiens and Hell, 2004; Taroni and Valentini, 2004; Diaspro *et al.*, 2005). Fluorescence microscopy utilizes fluorescently labeled probes of high biochemical affinity to image the molecular composition and dynamics of biological structures. Moreover, the use of probes that change their fluorescence properties in response to specific physiological parameters enables one to analyze the physiological state of cells or tissues (Birks, 1970; Emptage, 2001; Zhang *et al.*, 2002; Lippincott-Schwartz and Patterson, 2003; Stephens and Allan, 2003). Fluorescence is highly specific either as an exogenous label (e.g., 4′,6-diamidino-2-phenylindole (DAPI) bound to DNA) or an endogenous tracker [(e.g., autofluorescence of NADH, or visible fluorescent proteins such as green fluorescent protein (GFP)] providing spatial and functional information through precise photophysical properties such as absorption, emission, lifetime, and anisotropy. Furthermore, sample preparation is relatively simple, allowing non-invasive imaging and three-dimensional (3D) mapping within cells and tissues to be achieved by means of computational optical sectioning, confocal laser-scanning microscopy (CLSM), and two-photon excitation microscopy (TPEM) (Periasamy, 2001). In particular, CLSM and TPEM are two comparatively recent fluorescence microscopy techniques that have improved the quality of biological images (Wilson and Sheppard, 1984; Denk *et al.*, 1990; Pawley, 1995a; Diaspro, 2002, 2004; Matsumoto, 2002; Amos and White, 2003).

The main value of both CLSM and TPEM is their ability to produce optical section images through very thick biological specimens, such as embryos or tissue slices. As these images exclude optical information from out-of-focus planes, they can be combined to make a 3D reconstruction of the specimen (Kriete, 1992; Bonetto *et al.*, 2004). The ability of fluorescence microscopy to detect specific molecules at very low concentrations depends strictly on the performance of fluorophores used. Fluorescent dyes featuring high extinction coefficients, high quantum yields, and large Stokes shift are now widely available. However, one must remember that their photostability is another attribute of crucial importance. In CLSM, where the fluorescence signal is collected from only a thin layer of the sample, the laser beam must be intense enough to obtain an adequate signal to form an image with a good signal-to-noise ratio in a reasonable scan time. Under these imaging conditions, the fluorescence emitted is often observed to decrease substantially with time, a phenomenon referred to as photobleaching. This shows that in CLSM the photostability problem can have a severe impact on the image formation process. Even in TPEM, despite the tight confinement of the excitation volume and the use of infrared light, the high peak power of the laser pulses may cause photodamage of the fluorescent probes being used (Brakenhoff *et al.*, 1996; Mertz, 1998; Patterson and Piston, 2000; Dittrich and Schwille, 2001; Konig and Tirlapur, 2002). In general, the loss of fluorescence that comes along with photobleaching has the undesirable effect of reducing the signal-to-noise ratio and the quality of the collected images and data (Pawley, 1995b).

On the other hand, it is worth noting that photobleaching has been exploited since the mid-1970s (Peters *et al.*, 1974; Poo and Cone, 1974; Edidin *et al.*, 1976; Axelrod *et al.*, 1976) for producing valuable information about biological system dynamics and the advent of GFPs (Tsien, 1998) has led to renewed interest in photobleaching experiments including the utilization of selective photobleaching for information encoding (Cole *et al.*, 1996; White and Stelzer, 1999; Reits and Neefjes, 2001; Davis and Bardeen, 2002; Braeckmans *et al.*, 2003; Braga *et al.*, 2004; Delon *et al.*, 2004; Stavreva and McNally, 2004; Straub, 2003). Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are the most popular photobleaching-based techniques (McNally and Smith, 2002) and are technically based on the ability of the confocal and multi-photon microscopes to confine photobleaching to an arbitrary pattern or region of interest. Unfortunately, the mechanism of photobleaching during confocal or multi-photon imaging is not fully understood and some aspects still remain obscure, even in the case of very popular molecules such as fluorescein in solution (Song *et al.*, 1995).

As lack of signal limits the precision of most fluorescent techniques and as bleaching and phototoxicity limit the available signal, we feel that photobleaching is one of the most important factors restraining future developments in all these fields. Although we are not yet in the position of being able to develop dyes that do not bleach, any attempt to do so must start with an effort to assemble what we know so far. This chapter attempts to do just that. We will attack this topic using both microscopic imaging and single-molecule studies under confocal and multi-photon excitation conditions.

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PHOTOBLEACHING

Fluorescence emitted by almost all fluorescent dyes fades during observation. This phenomenon is called photobleaching or dye photolysis[1] and involves a photochemical modification of the dye resulting in the irreversible loss of its ability to fluoresce. Even though, in some cases, a fluorescent molecule can be switched on again after an apparent loss of emission ability or from a natural initial dark state (photocycling) or is able to switch on and off by itself (blinking) within a short timescale, we refer to photobleaching as an irreversible phenomenon.[2] This point is particularly relevant in FRAP and FLIP because these techniques are rigorously based on the idea that fluorescence recovers only because fluorescent molecules diffuse into the bleached sample volume. In this case, it is even more important to be clear that the photobleached state cannot be reversed.

Apart from this warning, we focus now on how photobleaching affects the image formation process. Indeed, fluorescent dyes in the excited state may undergo chemical or biochemical reactions that lead to their rapid degradation and destruction and consequent loss of image quality during measurement. In a certain sense, fluorophores are consumed by being observed. When fluorescent molecules are illuminated at a wavelength for which they exhibit a "good" cross-section (Weber and Teale, 1958; Chen and Scott, 1985; Tsien and Waggoner, 1995; Harper, 2001; Xu, 2002), possibly close to the cross-section maximum, there is a shift from the ground energy level (S) to the singlet-excited energy level (S*). Such a temporary excess of energy can be dissipated by the emission of fluorescence, or in radiationless processes such as internal conversion and intersystem crossing to the excited triplet state (T*). The decay times from S* and T* to S are different according to the selection rules, and are of the order of 1 to 10 ns and 10^{-3} to 10^{-6} s, respectively.

However, a molecule in the excited singlet or triplet state can also undergo a permanent structural change that often annihilates its ability to fluoresce, thereby becoming a photobleached molecule. Many factors, such as the molecular environment and the intensity of excitation light, may affect the mechanism, and thus the reaction order and the rates of photobleaching (Bernas *et al.*, 2004). In fact, spatial variability in the photobleaching process has been demonstrated within individual cells (Benson *et al.*, 1985). Figure 39.1 demonstrates an example, where Acridine Orange bound to DNA and RNA (green fluorescence and red luminescence,[3] respectively) of the same cell bleaches at different rates. This dye binds to double-stranded nucleic acids by intercalation, while stacks are formed on single-stranded nucleic acids. Apparently these two forms of the same dye exhibit different photostability under the same illumination conditions.

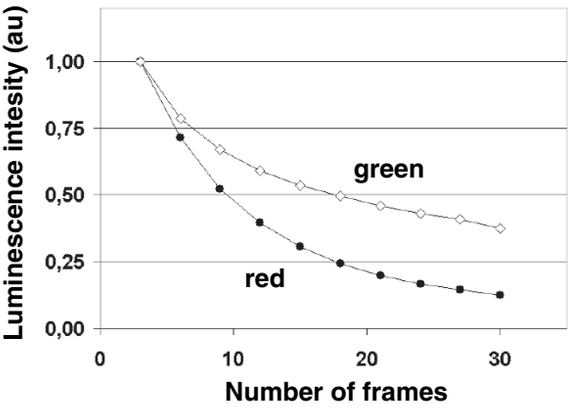

FIGURE 39.1. Loss of luminescence intensity of Acridine Orange bound to DNA in the nucleus and RNA in the cytoplasm of a fixed human fibroblast during acquisition of subsequent confocal frames. The initial intensities of luminescence in two areas of nucleus and cytoplasm were normalized. Images collected on Bio-Rad MRC1024, simultaneous excitation 488 nm, 0.5 mW at the specimen, dichroics 527DRLP, 565DRLP, emission filters 540/30nm and RG630, lens 60× NA 1.4 PlanApo (Zarebski and Dobrucki, unpublished; see also Dobrucki and Darzynkiewicz, 2001; Bernas *et al.*, 2004, 2005, for further information on the kinetics of this process and the way to minimize the differential photobleaching).

Figure 39.2 shows a similar effect obtained using two-photon excitation in the picosecond regime. Even if the sample is relatively thin, significant photobleaching occurs under two-photon excitation after delivering a larger dose of light.

Photobleaching Mechanisms

Many phenomena, such as self-quenching or fluorescence (or Förster) resonance energy transfer (FRET), are known to affect the amount of light emitted by an excited fluorescent molecule. The manner in which these processes reduce the fluorescence intensity is relatively well understood. In contrast, photodestruction is still a poorly understood phenomenon and few data are available on the bleaching properties of most common fluorophores. Dye photolysis is a very complex process.

Several theories have been proposed to explain photobleaching. The main causes seem to involve photodynamic interactions between excited fluorophores and molecular oxygen (O_2) in its triplet ground state and dissolved in the sample media. If the dye has a relatively high quantum yield for intersystem crossing, a significant number of dye molecules may cross from a singlet excited state S* to the long-lived triplet excited state T*, a process that permits these molecules to interact with their environment for a much longer time (milliseconds instead of nanoseconds). Interactions between O_2 and dye triplets may generate singlet oxygen according to $T^* + {}^3O_2 \rightarrow S + {}^1O_2$. Singlet oxygen has a longer lifetime than the excited triplet states of the dyes. Moreover, several types of damaging oxygen free radicals can be created when it decays. A fluorophore in the excited triplet state is also highly reactive and may undergo irreversible chemical reactions involving other intracellular organic molecules. All these chemical reactions depend both on the intracellular singlet oxygen concentration and on the distance between the dye and intracellular components such as proteins, lipids, etc. Therefore, the number of photons emitted before a dye molecule is destroyed depends both on the nature of the dye molecule itself and on its environment.

[1] Photobleaching is the process whereby a molecule is rendered nonfluorescent. This may be caused by photolysis (lysis, breaking open) or other mechanisms such as bonding of excited fluorophores to nearby molecules. Photo-oxidation describes the first stage of many such processes.

[2] We should also note here another confusing parameter. If a macromolecular target is over-labeled in the sense of being bound to more than the optimal number of dye molecules, then they may tend to quench each other and so reduce the total emission. Under these conditions, bleaching some dye molecules may actually increase total light output from the preparation because the bleached molecules no longer quench.

[3] Because the red component of Acridine Orange (AO) emission has a long time component, it is more properly called luminescence. In a single-beam confocal, only the fast component is seen because the signal can only reach the PMT for a few microseconds.

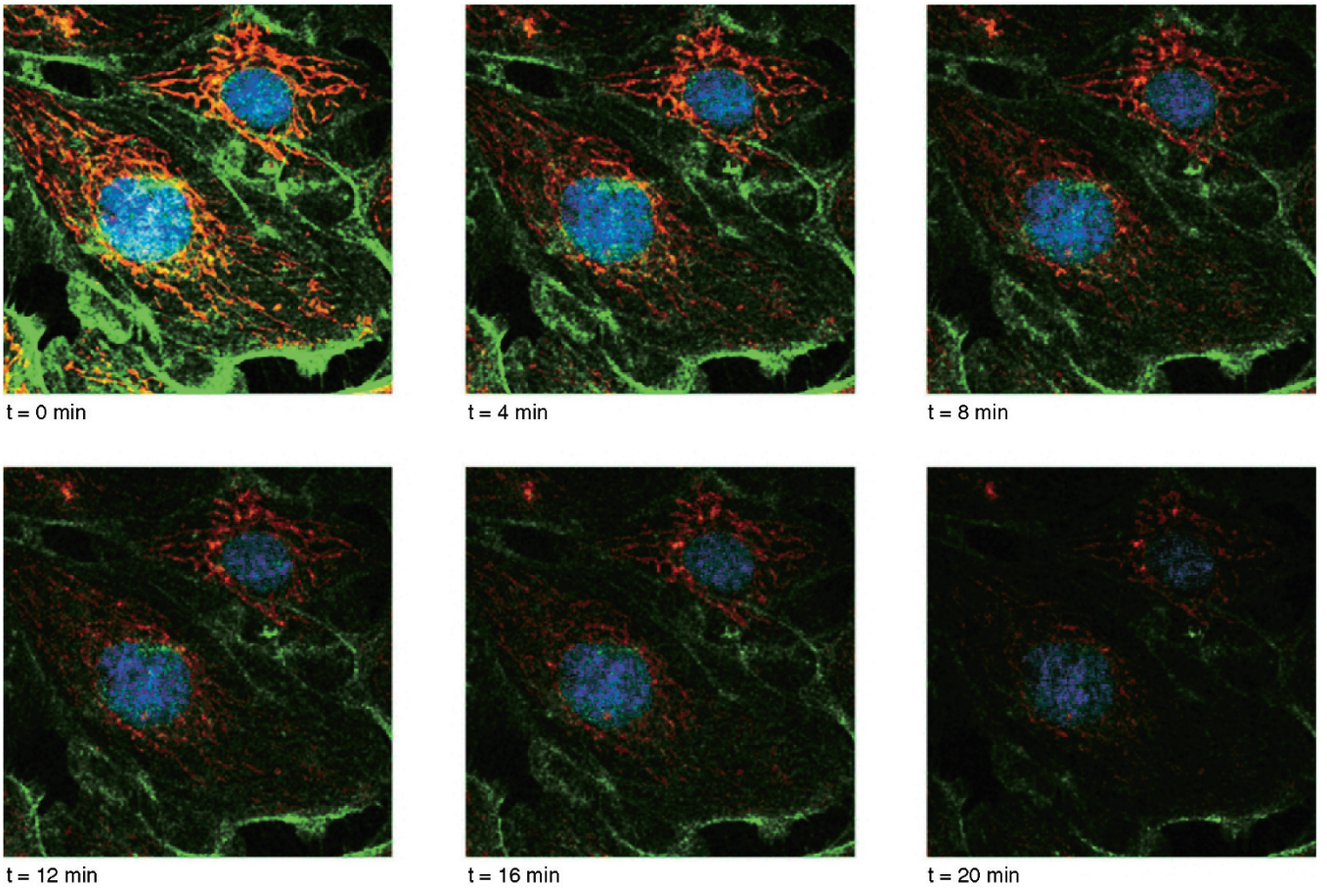

FIGURE 39.2. Loss of fluorescence intensity of three probes, bound to DNA (DAPI, blue), mitochondria (MitoTracker Red CMXRos, red), and actin filaments (BODIPY-FL phallacidin, green) in fixed bovine pulmonary artery endothelial (BPAE) cells. Two-photon excitation was provided by a Leica TCS SP2 AOBS confocal microscope coupled to a Chameleon-XR (Coherent, Santa Clara, CA) ultrafast tunable titanium–sapphire laser through the IR port. Pulse width was increased to picoseconds from 140 fs, 760 nm wavelength, 20 mW average power at the entrance of the scanning head. Temporal series was acquired at a rate of 729 ms per image every 6 s. Image dimensions are 512 × 512 pixels over a 79.76 × 79.76 μm^2. (Images collected by Paolo Bianchini, LAMBS-MicroScoBio, University of Genoa, http://www.lambs.it.)

Some fluorophores have a very short useful lifetime, fading after the emission of only a few hundred photons, whereas other molecules in other surroundings can emit a very large number of photons (tens of millions) before being bleached. If a dye is protected from reaction with environmental molecular oxygen, the observed rate of photobleaching is lower (Bernas *et al.*, 2004). This occurs naturally in GFP (Tsien, 1998), where the intrinsic chromophore is positioned in the core of a β-barrel structure (Ormo *et al.*, 1996).

In addition, either multi-photon events or the absorption of a second photon by a molecule already in an excited state may also be involved in the photobleaching of some dyes. Because of the low absorption cross-section and its quadratic dependence on the incident power, multi-photon excitation requires an excitation intensity several orders of magnitude higher than that needed for one-photon excitation. Unfortunately, this increases photobleaching because of the high probability of photochemical degradation in the long-lived triplet state and the interplay of multi-photon ionization processes (Dittrich and Schwille, 2001; Michalet *et al.*, 2003).

On the other hand, as excitation only takes place in a tightly confined volume of the sample, bleaching away from the plane of focus is essentially eliminated and the overall photobleaching and photodamage within the whole sample is usually less (Bianco and Diaspro, 1989; Denk *et al.*, 1990; Diaspro, 2004). Moreover, the tightly controlled excitation volume can allow very precise control of intentional photodamage that might be used for uncaging or even microsurgery when needed (Diaspro *et al.*, 2003; Chapter 38, *this volume*).

Until now, the photochemical reactions responsible for the photobleaching have been investigated in greatest detail for the dye fluorescein. Bleaching can be described in terms of a photobleaching quantum efficiency Q_b, defined as the ratio between the number of molecules that have been bleached and the total number of photons absorbed during the exposure period. The average number of photons emitted before a dye molecule bleaches is the ratio between the fluorescence quantum yield Q_f of the dye and Q_b. At excitation intensities of 10^{23} to 10^{24} photons $cm^{-2} s^{-1}$, approximately corresponding to 1 mW of power incident on a spot of 0.25 μm radius at 488 nm wavelength (Schneider and Webb, 1981), fluorescein bleaches with a $Q_b \approx 3 \times 10^{-5}$. Therefore, during its useful lifetime the average fluorescein molecule dissolved in water emits 30,000 to 40,000 photons before being permanently bleached (Hirschfeld, 1976; Mathies and Stryer, 1986; Tsien and Waggoner, 1995).

Both the experiments and the mathematical models used to investigate the behavior of free and bound fluorescein have demonstrated that, in fluorescence microscopy, its photobleaching cannot be described by a single-exponential process. However, the single-exponential process is appropriate for the special case when the photobleaching reaction is primarily between the triplet state of the dye and molecular oxygen (Hirschfeld, 1976; Song *et al.*, 1995).

Although quenching of the triplet excited state (that forms a semi-oxidized radical form of the dye) by mercaptoethylamine restores fluorescein molecules to their singlet ground state, and reduces photobleaching (Song *et al.*, 1996), there is no general agreement on the energy states involved in photobleaching. In single-molecule detection conditions, evidence of a two-step photolysis has been reported for several coumarin and rhodamine derivatives (Eggeling *et al.*, 1998). These authors suggest that excited triplet states T_n, higher in energy than T*, can occur when two, distinct, successive photon absorptions occur at high irradiance. Excitation at low irradiance yields longer survival times because, at any instant, fewer molecules are in the T* state to be excited to T_n (Eggeling *et al.*, 1998; Deschenes and van den Bout, 2002).

Fluorescence correlation spectroscopy has been used to measure and characterize the photobleaching of rhodamine 6G and FITC in different solutions (Widengren and Rigler, 1996; Delon *et al.*, 2004).

The high photon flux used in two-photon excitation microscopy seems to lead to higher-order photon interactions. The dependence of fluorescence intensity and photobleaching rate on excitation power have been studied for Indo-1, NADH, and aminocoumarin under one- and two-photon excitation (Patterson and Piston, 2000). The results of these studies suggest that higher-order photobleaching is common in two-photon excitation microscopy. Therefore, the advantage of reduced photobleaching associated with the limited excitation volume, characteristic of TPEM in thick samples, is offset by greater in-focus damage, making the technique less suitable for studying thin specimens. A comparison of photobleaching effects under single- and multi-photon excitation has been performed in plant cells (Kao *et al.*, 2002). At the laser intensities used to study chloroplasts in the protoplasts from *Arabidopsis thaliana*, the photobleaching cross-section under two-photon excitation is low, requiring the high peak power levels common in TPEM. This generates greater localized photobleaching effects than using conventional excitation (Kao *et al.*, 2002). However, reducing specimen exposure allows one to obtain significantly more signal by multi-photon than by one-photon excitation in high-resolution optical sectioning of thick samples (Drummond *et al.*, 2002). In addition, the use of phase-optimized, shaped pulses in two-photon excitation microscopy has been demonstrated to attenuate the photobleaching of a GFP variant by a factor of 4 (Kawano *et al.*, 2003).

Intrinsically fluorescent proteins such as the phycobiliproteins (PBP) generate reactive oxygen species (ROS) in living cells. Photoactivation of R-phycoerytrin, C-phycocyanin, and allophycocyanin by visible light results in the generation of both singlet oxygen and superoxide. This contributes to both the photobleaching and the phototoxicity of PBPs *in vivo* (He *et al.*, 1997). In addition, flavins and flavoproteins excited to a triplet state by violet-blue light can be reduced by endogenous cellular reducing agents, resulting in the production of H_2O_2 in cultured mouse, monkey, and human cells (Hockberger *et al.*, 1999). However, imaging the same cells by TPEM at 1047 nm did not induce measurable production of H_2O_2 (Hockberger *et al.*, 1999). Although the molecular oxygen present in the surrounding environment does not interact directly with the chromophore of GFP, photoactivation is thought to generate endogenous singlet oxygen, which induces damage to this chromophore, as demonstrated in transfected COS7 kidney cells and *E. coli* bacteria (Greenbaum *et al.*, 2000). In BY-2 tobacco cells loaded with oxidation-sensitive dyes, excitation light induces the production of ROS. The amount of ROS increases if the cells express an intrinsically fluorescent protein that absorbs the excitation light (Dixit and Cyr, 2003). This process results in an increase in mitotic arrests and shows a nonlinear relationship to the excitation light intensity (Dixit and Cyr, 2003). The role of molecular oxygen in phototoxicity exerted by extracellular fluors has been demonstrated in animal and plant cells (Dobrucki, 2001). The involvement of oxygen in photobleaching processes is supported by data, which demonstrate that anoxia significantly reduces photobleaching rates of propidium and chromomycin A_3 bound to DNA (Bernas *et al.*, 2004).

Apart from fluorescent proteins, nonlinear multi-photon excitation in the near-infrared of a variety of endogenous absorbers, such as water and NAD(P)H, can induce the formation of ROS resulting in oxidative stress (Konig and Tirlapur, 2002).

Reducing Photobleaching

The prevention of the fading of fluorescence emission intensity is very important not only for quantitative microscopy but also for obtaining high-quality images. Unfortunately, reducing photobleaching by decreasing the excitation time or by lowering the excitation intensity leads to a reduction of the fluorescence signal. As lower signal increases the effect of Poisson noise and obscures low-contrast features, deciding on the optimal excitation level is always a trade-off in which ideally one reduces the light dose until one can no longer see the details one needs to understand. Optimizing the excitation intensity can be beneficial in 3D imaging (Bernas *et al.*, 2004).

A good strategy starts with the selection of a fluorophore with the high photostability. Figure 39.3 (A,B) shows a comparison of the results of photobleaching of four fluorescent probes associated with chromatin in different preparations of fixed HeLa cells. The probes differ in molecular structures and mechanisms of binding to chromatin. The same dose of light delivered at similar photon fluxes resulted in dramatically different loss of fluorescence signal [Fig. 39.3(A)] and a different number of total photons recorded (Fig. 39.3(B)]. The choice of the best fluorophore is not trivial, however. In this example, one might draw the conclusion that propidium, a DNA intercalator, is the best choice for imaging chromatin. In fact, eGFP fused with histone H2B gave the best results in this experiment because eGFP yielded reasonable images even at an excitation photon flux of 0.17 W/cm^2, where photobleaching was negligible. Thus, the total number of photons emitted by GFP before bleaching was the greatest. The other probes tested required higher intensities of excitation light, bleached at greater rates and provided less total photons (Bernas *et al.*, 2004).

As molecular oxygen is one of the main actors in photobleaching, deoxygenating the specimen often improves the survival period of both the dye and the cell. In living cells or tissues that tolerate anoxia, or even hypoxia, oxygen may be removed by bubbling N_2 through the media or by using biological oxygen scavengers (Bloom and Webb, 1984; Dobrucki, 2001). Chemicals capable of quenching singlet oxygen can also be employed to reduce the effects of photobleaching. Carotenoids such as as crocetin and etretinate are good singlet oxygen quenchers in cell cultures (Kohen *et al.*, 1986; Reyftmann *et al.*, 1986; Manitto

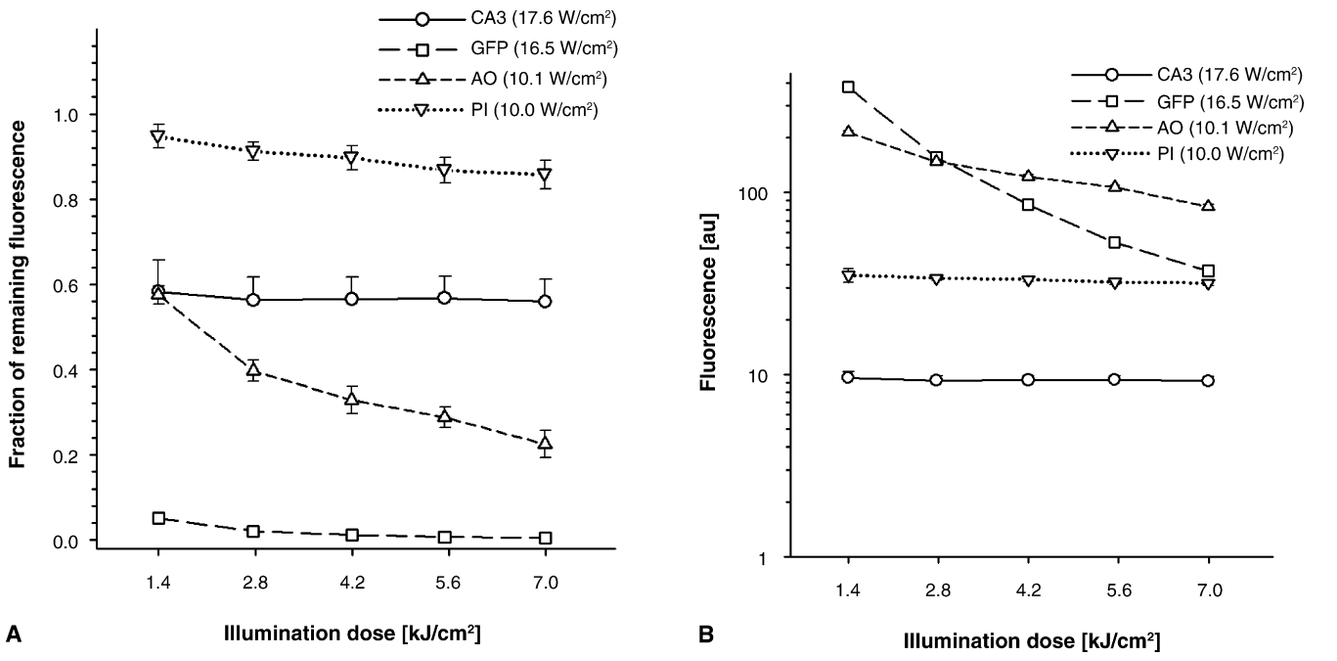

FIGURE 39.3. Loss of signal of four fluorescent probes bound to chromatin due to photobleaching by exciting light. Fraction of fluorescence remaining (A) and the total number of photons detected per one pixel (dwell time 13.3 µs; B) after delivering increasing doses of excitation light is shown for propidium (PI), Acridine Orange (AO, green fluorescence of intercalated monomers), chromomycin A_3 (CA3), and eGFP fused to histone H2B. The conditions of the experiment are described elsewhere (Bernas *et al.*, 2004, 2005).

et al., 1987). Other oxygen-reactive protective molecules include vitamin E analogues, vitamin C, reduced glutathione, imidazole, cysteamine, and the amino acid histidine (Tsien and Waggoner, 1985).[4]

Figure 39.4 compares the photobleaching kinetics of propidium bound to the DNA of fixed human fibroblasts, equilibrated with air or argon, as a function of the excitation dose in the range from 0 to 50 kJ/cm^2 and it shows that argon lessens signal loss resulting from photobleaching. Figure 39.5 shows the fraction of fluorescence that remains unbleached after the same type of cells have received the same total dose of light at different photon fluxes in the range from 1 to 111 W/cm^2, respectively. It demonstrates how a given dose of illumination causes less bleaching when delivered at a low photon flux, or under argon (Bernas *et al.*, 2004). Figure 39.6 shows the potential of anoxic imaging in protecting fluorescence and facilitating collection of full 3D datasets (after Bernas *et al.*, 2004).

Other common antifade reagents are diazabicyclo-2,2,2-octane (DABCO), *n*-propyl gallate and *p*-phenylenediamine. 2-mercaptoethylamine is used mainly when observing chromosome and DNA specimens stained with propidium iodide, Acridine Orange, or chromomycin A_3.

Although the anti-fade agents, whose effects are diagrammed in Figure 39.7, are highly efficient in retarding photobleaching, each has side effects. One of the most effective antifade reagents, *p*-phenylenediamine (PPD), suffers from thermosensitivity, autofluorescence, and cell toxicity, making it unsuitable for *in vivo* studies (Bock *et al.*, 1985; Krenik *et al.*, 1989). *N*-propyl gallate (NPG) is both photo- and thermostable. While not as effective as PPD, it can be used for *in vivo* studies. However, although NPG is very effective in retarding bleaching in FITC-stained Reh6 cells, it reduces the initial value of fluorescence intensity both in conventional epi-fluorescence and in confocal microscopy (Souchier *et al.*, 1993). Comparing NPG with other anti-fading agents, these authors conclude that the choice of an anti-fading medium depends on the desired results: a slower decay of fluorescence combined with initial quenching versus a faster photobleaching rate but starting at a higher initial emission intensity.

An extensive comparison among different homemade and commercial anti-fading media has been performed by CLSM in NIH 3T3 cells stained with FITC-phalloidin (Ono *et al.*, 2001). These authors use the equation $EM_t = (EM_0 - B)e^{-t/A} + B$, where EM_t is the emission intensity at time t, B the background intensity, and A the anti-fading factor, that is, the time spent until fluorescence intensity decays to $1/e$ of EM_0. As a general rule, for the media examined (SlowFade and ProLong, Molecular Probes, Inc., Eugene, OR; PermaFluor, Lipshaw/Immunon, Pittsburgh, PA; FluoroGuard, Bio-Rad Labs, Hercules, CA), and in agreement with Souchier and colleagues, they found that high initial emission intensities are accompanied by faster decreases of fluorescence. With bright and photostable molecules, such as the Alexa dyes (Landon, 1997), media that include an anti-fading agent are a good choice, even if they have a lower initial fluorescence intensity.

Very recently, a new family of synthetic nanoparticle fluorophores has been introduced. They mainly consist of a core of cadmium selenide coated with zinc sulfide. These nanocrystals, named Quantum Dots (Quantum Dot Corporation, Hayward, CA) have a large Stokes shift and are very bright and photostable (Hines

[4] To be effective as radical scavengers, these agents must be used at concentrations high enough to be incompatible with live-cell studies. Even cells that do seem to not die in terms of popular viability assays often suffer other types of damage that may affect their physiology sufficiently to preclude acquisition of relevant information. See also Chapter 38, *this volume*.

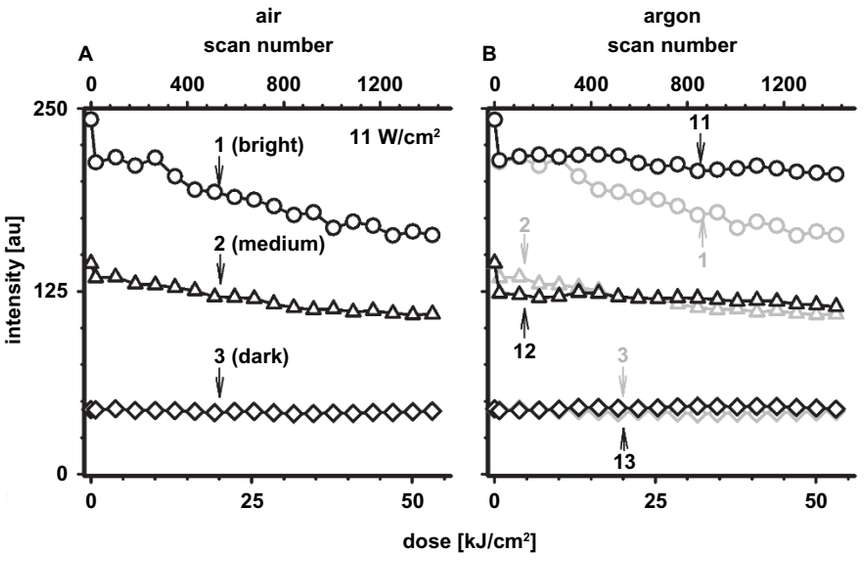

FIGURE 39.4. Kinetics of photobleaching of propidium bound to DNA, in air or argon. Intensity (0–250 a.u.) versus dose (0–50 kJ/cm^2). Fixed cells were stained with propidium, and equatorial sections scanned with the laser in a confocal microscope under air (A) or argon (B). Twenty scans were accumulated to form one image, and the average intensity (arbitrary units; a.u.) of pixels of different initial brightness in images is plotted relative to the accumulated dose of light. The flux of the incident light is indicated. (A) Bright pixels (curve 1) are bleached more rapidly than darker ones (curves 2, 3). Curves for only three of the 25 brightness classes are shown for clarity. The results are the means of five experiments. (B) Argon lessens photobleaching. Curves 1 to 3 from (A) are included for comparison with their counterparts (i.e., 11–13) in argon (after Bernas *et al.*, 2004).

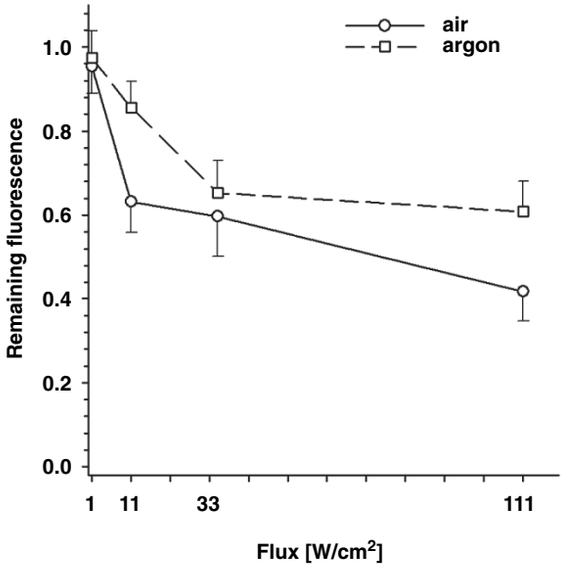

FIGURE 39.5. Analysis of photobleaching of propidium bound to DNA in air and argon. The fraction of fluorescence remaining after exposure to the same total dose of exciting light, but delivered by laser beams of different photon fluxes. Laser intensity was adjusted using neutral density filters and the total time of illumination was adjusted to deliver the same total light dose to each sample. Error bars: 95% confidence intervals (after Bernas *et al.*, 2004).

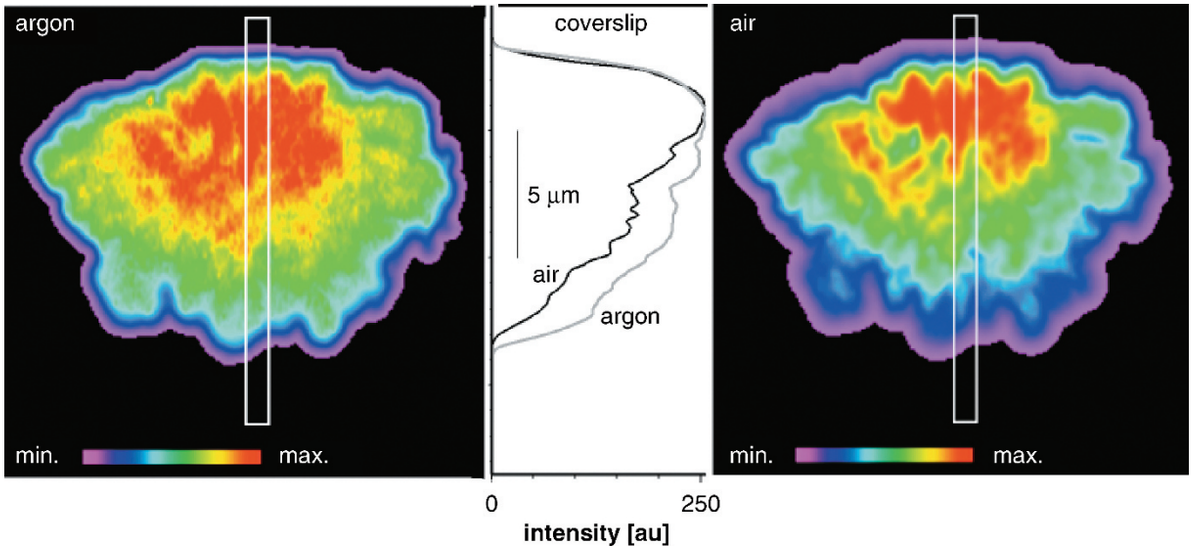

FIGURE 39.6. An *xz*-projection from 3D images collected in argon (left) or in air (right) of chromatin in a cell in mitosis, with condensed chromosomes stained with propidium. Sixty-nine consecutive images were collected at a 330 nm *z*-spacing using an average excitation flux of 11 W cm^{-2} in the focal plane and starting near the coverslip. Maximum intensity projections (right and left) and fluorescence (center) measured along the white vertical bar. The intensity drops off more rapidly as one moves away from the coverslip under air because of increased photobleaching (after Bernas *et al.*, 2004).

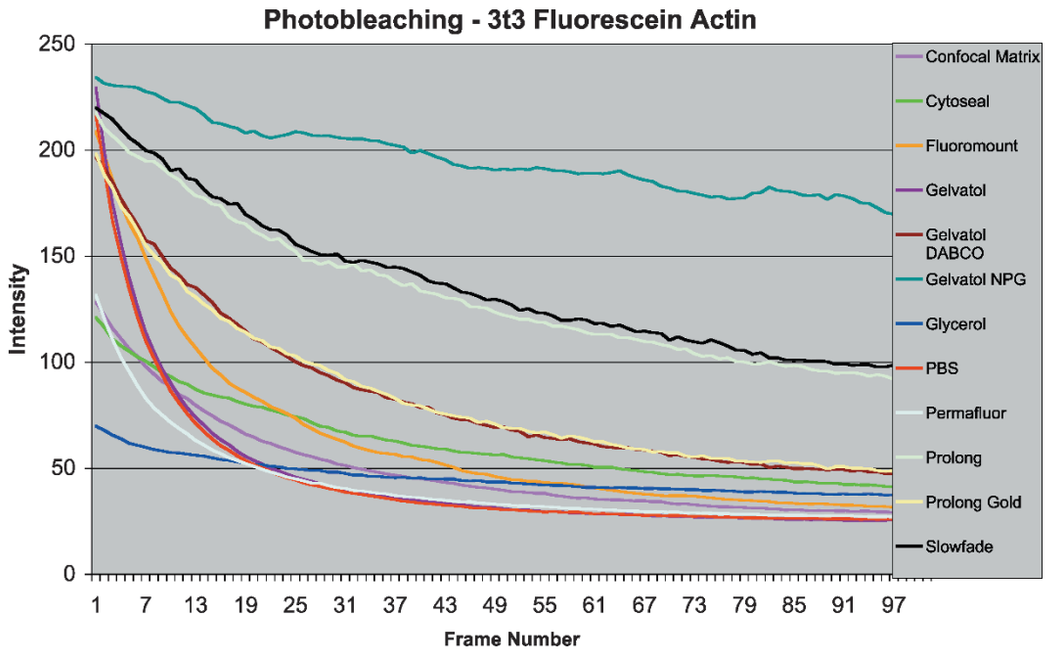

FIGURE 39.7. Loss of fluorescence intensity due to photobleaching in similar samples submerged in physiological saline or in mounting media supplemented with various commercial anti-fade agents. The efficiency of countering photobleaching and a loss of the initial signal varied widely among different formulations. Samples: fixed FoLu lung fibroblasts, actin stress fibers stained with phalloidin-fluorescein. (Data kindly provided by M.W. Davidson.)

and Guyot-Sionnest, 1996; Mattoussi *et al.*, 2000; Watson *et al.*, 2003; Jaiswal and Simon, 2004). A surface polymer layer enables conjugation of Quantum Dots to antibodies and other biomolecules (Wu *et al.*, 2003), allowing the development of new immunofluorescence protocols that take advantage of their fluorescent properties (Ness *et al.*, 2003). Quantum Dots allow multicolor imaging in demanding biological environments such as in living animals or organs and within cellular cytoplasm (Sokolov *et al.*, 2003; Larson *et al.*, 2003; Jaiswal and Simon, 2004). These fluorescent probes exhibit two-photon cross-sections of the order of thousands of Goeppert-Meyer units (GM), ($1\,GM = 10^{-50} cm^4 s/photon$) and a wide range of emission wavelengths (Diaspro *et al.*, 2005). They should be good candidates for deep, multi-color imaging in biological and medical applications (Sokolov *et al.*, 2003; Voura *et al.*, 2004). On the other hand, some problems in using Quantum Dots relate to their relatively large size and mass, their intermittent fluorescent behavior, their high propensity for aggregation, and the fact that their high cross-section and relatively long singlet-state lifetime, cause them to reach singlet-state saturation at relatively low power levels in scanned-beam microscopy.

PHOTOBLEACHING AT THE SINGLE-MOLECULE LEVEL

Any single-molecule spectroscopy experiment stops when the probe molecule photobleaches. This limitation becomes crucial when the time scale of the biological process under examination is comparable to the survival time for the molecule itself (Deschenes and van den Bout, 2002). In certain matrices and at low temperature, photobleaching can be delayed almost indefinitely (Basché, 1998). However, under usual laboratory conditions (room temperature, in atmosphere) each molecule can emit a finite, though sometimes very large (Deschenes and van den Bout, 2002), number of photons.

Single-molecule photobleaching can be defined as a permanent transition to a dark electronic state that occurs typically after $N \sim 10^6$ to 10^8 photon absorption events. This implies a few seconds of observation at an average emission of 10^6 fluorescence photons/second, and leads to significant signal-to-noise challenges for single-molecule spectroscopists.

In fluorescence laser scanning microscopy, excitation volumes range from 0.1 to $1\,\mu m^3$. For molecular concentrations of about $100\,nM$ to $1\,\mu M$ (i.e., similar to that of most of the protein systems in cells), the number of fluorescent emitters in the excitation volume varies from 10 to 1000. This number lies between the single-molecule and the bulk level. Fading of images of cells and tissues under repeated imaging in fluorescence CLSM or TPEM, can be ascribed to: (1) a change in the number of fluorophores present (single-molecule bleaching), (2) a variation of the fluorescence quantum yield, and (3) a change in the fluorescence emission dynamics (single-molecule blinking).

The first mechanism leads to photobleaching when viewed at the bulk level. The second mechanism can be due to chemical reactions (e.g., photo-induced protonation/deprotonation) or to photo-induced transitions to other electronic states. At the bulk level, an overall decrease in the fluorescence quantum yield can also be due to an increase in the rate of reversible transitions between a bright and a dark state of the individual molecules, a phenomenon known as blinking. All these situations may lead to a decrease in overall fluorescence emission under imaging conditions.

In general, blinking characteristic times usually occur on a time scale of 1 to 100 ms, but they can also extend to much longer intervals. Fluorescence emission can sometimes recover after minutes to hours making it difficult to discriminate between permanent bleaching and blinking.

Photobleaching of Single Molecules

Not much data and few systematic studies are available on single-molecule photobleaching, a process that is usually characterized, at the time resolution of CLSM or TPEM, by a steep drop of the fluorescence emission to the background level. The observation of such a sharp transition is indeed used as a fingerprint of the single-molecule emission and has been used to discriminate single-molecule spots in fluorescence images from those representing aggregates (Chirico *et al.*, 2001, 2002; Diaspro *et al.*, 2001; Maher *et al.*, 2002; Cannone *et al.*, 2003). Photobleaching also affects other single-molecule spectroscopic approaches such as surface-enhanced resonant Raman spectroscopy, a process recently highlighted by Maher and colleagues (2002).

The photophysical origin of single-molecule photobleaching has been investigated on several dyes used for microscopy. One of the first attempts at a systematic study dealt with a series of four dyes, pyrene, rhodamine 6G fluorescein and indo-1, spread by spin-coating on a chemically etched glass (see details in Chirico *et al.*, 2001, 2003).[5] For the two-photon excitation used in the study, the authors found a power law dependence of the bleaching rate on the excitation power with an exponent ≈2.5. This finding proved that increasing the excitation power does not imply a gain in the total fluorescence photon flux. Because the fluorescence emission depends on the second power of the excitation intensity, the dependence of the total number of photons emitted versus the excitation intensity, for TPE, scales as $\sim I^{-0.5}$, where I is the excitation intensity. Moreover the bleaching rate depends on the duty cycle of the excitation. If continuous excitation is replaced by pulsed excitation without increasing the peak brightness, the bleaching rate diminishes linearly with the excitation duty cycle (the ratio of the time the molecules are irradiated to that when they are not irradiated). A marked dependence of the bleaching rate on the substrate temperature was also reported.

Recently, Deschenes and van den Bout (2002) reported a detailed single-molecule study of rhodamine 6G molecules embedded in a film of poly(methylacrylate) (PMA) under vacuum and using very low excitation intensities, between 2 W/cm^2 and 10,000 W/cm^2. The authors reported a remarkable decrease of the photobleaching rate at low excitation intensities and an increase in the total number of photons emitted up to $\sim 10^9$ photons per molecule before bleaching. They propose a four-state model, sketched in Figure 39.8, that accounts for the observed nonlinear intensity dependence of the photobleaching rate. This model seems to fit most of the single-molecule photobleaching studies published up to now.

Photobleaching causes the fluorescent output from a group of dye molecules to decay in a manner described by either a single- or a multi-exponential function of the total accumulated exposure (Xie and Trautman, 1998; Widengren and Riegler, 1996; Eggeling *et al.*, 1997, 1998; Wennmalm and Riegler, 1999; Ko, 2004).

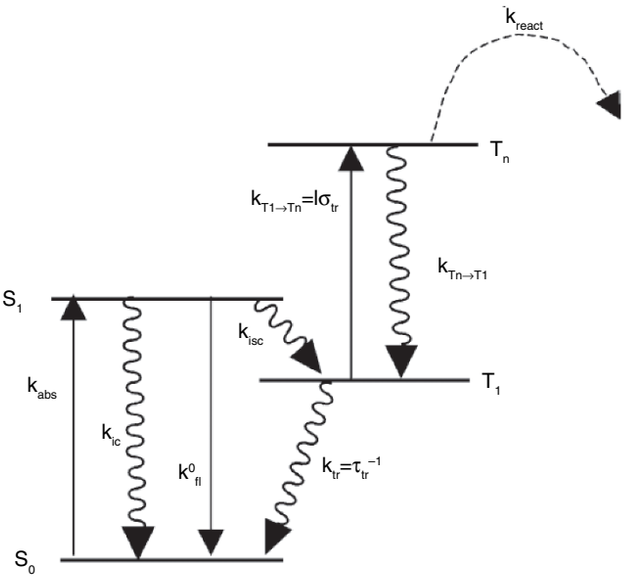

FIGURE 39.8. Perrin–Jablonski diagram of the relevant states for rhodamine 6G photobleaching. Absorption (k_{abs}) from S_0 to S_1 may lead to non-radiative internal conversion (k_{ic}), fluorescence (k_{fl}^0), or intersystem crossing (k_{isc}) to T_1. From T_1, the molecule may relax back to S_0 (k_{tr}), or absorb a second 532 nm photon ($k_{T1\rightarrow Tn}$) and go to T_n. From there, the molecule may either relax back to T_1 ($k_{Tn\rightarrow T1}$) or react (k_{react}), leading to irreversible photobleaching. (Derived from Deschennes and van den Bout, 2002.)

Multi-exponential distributions are typically found for ensemble measurements while single-exponential bleaching times are measured on a series of single, entrapped molecules.

The multi-exponential distribution of photobleaching times has been interpreted either as being caused by intrinsic heterogeneity of the biological sample (Xie and Trautman, 1998), or as an artifact caused by the use of non-uniform excitation profiles in the beams used to produce the bleach. Molski (2001), who calculated bleaching kinetics in single-molecule fluorescence spectroscopy using renewal theory, suggested that non-single-exponential distributions of bleaching times could be due to true heterogeneity in the sample (in-homogeneous broadening). He suggested a five-state model where bleaching occurs from the excited triplet states, and found an exact formalism to calculate distributions of the number of photon counts and the time before photobleaching. He found exponential distributions for the average bleaching number and the average bleaching time and related these two quantities to the transition rate constants. Berglund (2004) has extended these results by considering theoretical models of the decay, via photobleaching, of surface-immobilized fluorescent molecules excited by a spatially varying laser intensity profile. He shows that the fluorescence decay measured in a non-uniform excitation profile is always non-exponential because molecules near the edge of the spot, where the light is dimmer, bleach more slowly.

These results suggest that the observation of multiple exponentials in the distributions of single-molecule photobleaching times could arise at least partially as an artifact of a spatially varying laser profile rather than indicating any heterogeneity in the bleaching process. Experimental support of this study appeared in an article by Ko (2004), who showed that, for rhodamine 6G in agarose gel, the bulk bleaching decays are described by a three-exponential decay while the single-molecule bleaching times are

[5] Solutions of various dyes were prepared as follows: (1) 100 m*M* rhodamine 6G in dimethylsulfoxide; (2) 100 m*M* fluorescein in TRIS buffer; (3) 100 m*M* pyrene powder in DMSO; (4) 100 m*M* Indo-1 powder in MilliQ water. The rhodamine and fluoroscein solutions were later diluted to 70 to 300 n*M* in ethanol before use. The glass slides were cleaned by being soaked in a 1% sodium dodecyl-sulfate for 24 h, then in a saturated NaOH-methanol solution for 2 h. The NaOH is removed by soaking the slides in 0.1% HCl for 2 h and then in diluted chromic solution (K_2CrO_4 in concentrated phosphoric acid) for 2 h. The slides were then stored in ethanol and finally rinsed thoroughly with MilliQ water and dried with nitrogen flow just before use. A drop of one of these work solutions was placed on a glass slide and spin-coated at 1000 rpm in a hood.

distributed according to a single-exponential function. It is encouraging that the average bleaching time they observed in bulk agrees very well with the mean of the single-molecule distribution. Rhodamine 6G entrapped in poly(vinyl alcohol) has been the object of another recent study of photobleaching at the single-molecule level (Zondervan *et al.*, 2004). These authors have investigated photobleaching as a function of illumination time, excitation intensity, the presence of oxygen, and temperature. They observed non-exponential kinetics related to primary photobleaching through two dark states — the triplet state and a radical anion — and to secondary photobleaching after the optical excitation of those dark states. The important claim of this study is that the presence of metastable states other than the triplet can drastically affect the photobleaching rate.

Photobleaching and Photocycling of Single Fluorescent Proteins

Among fluorescent biomolecules, single-molecule photobleaching studies have been reported on several variants of the GFP, and more generally, of the so-called visible fluorescent proteins (VFPs). Blue mutants, used in combination with yellow GFP were well known to cellular microscopists as a good donor–acceptor pair for measuring intracellular interactions via FRET. However, as blue GFP variants are generally dim (Rizzuto *et al.*, 1996; Cubitt *et al.*, 1997) and tend to photobleach readily (Ellenberg *et al.*, 1998), alternative multicolor pairs were developed. Of these, the most popular currently is cyan fluorescent protein (CFP) (Heim *et al.*, 1994) and the red-shifted yellow fluorescent protein (YFP) (Ormö *et al.*, 1996). CFP is brighter than BFP and is more photostable under imaging (Ellenberg *et al.*, 1998; see Chapter 45, this volume).

Spontaneous fluorescence recovery of YFP molecules from seemingly irreversible photobleaching using 405 nm excitation was reported very early in the GFP literature (Dickson *et al.*, 1997). The authors have called this effect optically induced switching. In fact, the chromophores of GFP occur in at least three protonation states. Two of them correspond to the cases in which the oxydril of the tyrosine 66 is protonated (neutral state) or de-protonated (anionic state). A third state corresponds to a zwitterionic case (Zimmer, 2002). Most of the observations on the recovery of GFP mutants after bleaching hint at the possibility that some of the presumably photobleached proteins were actually residing in a dark state caused by a neutral chromophore. The switching effect has an important impact on the suitability of GFP mutants for both cellular imaging and single molecule experiments. However, investigations on the widely used EGFP mutant showed no optical switching behavior under a variety of experimental conditions (Cinelli *et al.*, 2000).

It is possible to detect single-molecule signals with sub-millisecond time resolution using an avalanche photodiode, provided that a sufficiently high fluorescence photon flux can be established. Early confocal microscopy experiments on single molecules of the GFP mutant E222 by Jung and colleagues (1998) increased the time resolution to a minimum integration time of 10 ms, a limit more recently reduced to 800 µs (Garcia-Parajo *et al.*, 2000). These experiments showed that spontaneous recovery of fluorescence emission from single GFP molecules can be observed as long as 180 min after an apparent photobleaching event and that photoinduced blinking was also present. The blinking on-times measured for this mutant were dependent on the excitation power with values comparable to those previously reported (Dickson *et al.*, 1997; Moerner *et al.*, 1999; Peterman *et al.*, 1999). At moderate illumination intensities of 1.5 kW/cm^2, half of the molecules reside in a dark state (Garcia-Parajo *et al.*, 2000). It is worth noting that this high proportion is not caused by the comparatively small transition yield of $5 \cdot 10^{-6}$ into the dark state so much, as by the long lifetime of this state $t_{off} = 1.6$ s.

An enhanced YFP mutant, termed E^{2}GFP, has been obtained that might be used as a single-biomolecule optical switch (Cinelli *et al.*, 2001). As with most GFP molecules, prolonged or intense excitation results in photobleaching (at 476 nm in the case of E^{2}GFP). However, E^{2}GFP is the only known mutant in which irradiation of the dark photobleached state (at 350 nm) produces an excited state that photoconverts to an anionic B form, which is also fluorescent.

Recently, a TPE investigation (Chirico *et al.*, 2004) at the single-molecule level showed that single-molecule photobleaching increases with a power law of 2.4 and that the fluorescence recovery after bleaching can be induced also by near-infrared light at 720 nm (Fig. 39.9). Several characteristics of this infrared (IR)-induced recovery makes this mutant promising both for prolonged cellular imaging and for opto-electronic data storage applications. Recovery spectra are very narrow and their widths do not change appreciably with the excitation power, as shown in Figure 39.10. Maximum recovery efficiency occurs at 720 nm. This corresponds to twice the peak wavelength of the spectrum of the dark state obtained in single-photon experiments (Nifosì *et al.*, 2003). The fourth-power dependence of the recovery shown in Figure 39.10(B) indicates that two two-photon transitions are required to overcome the energy barrier from a dark state to the optically active form coupled to it. The second transition is likely to bring the system from the excited dark state (or its relaxed form) to a higher excited level. Detuning between the two transitions may cause the observed sharp recovery spectrum. Vibrational–electronic couplings could also contribute to reduce the width of the recovery spectrum.

Bleaching and Autofluorescence

A main concern about photobleaching at the single-molecule level stems from the effort to follow a single-protein system *in vivo*. The ability to do this would allow one to study in detail basic processes such as cellular internalization and cell trafficking by exploiting all the known photophysical properties of the probes being used. Although, within the nucleus, autofluorescence is only slightly above the level encountered in common gel samples, investigations in the cytoplasm are hampered by a significant level of background signal. It is therefore necessary to optimize the experimental conditions, including the choice of a fluorophore with minimal photobleaching, before single-molecule detection in living cells becomes conceivable. As an important step to this end, Kubitschek and co-workers have again employed YFP proteins. The authors investigated the diffusion of the S65G/S72A/T203F mutant in glycerol/water mixtures (Kubitschek *et al.*, 2000). They were able to track single YFP molecules with a time resolution of 10 ms, a signal of 8 ± 3 counts/pixel above background, and a signal-to-noise ratio of 4. Photobleaching was still a limiting factor, yielding, on average, a maximum of only 10^5 emitted photons and an observation time of 100 ms.

Other Fluorescent Proteins

Besides GFPs, the light harvesting protein systems of photosynthetic organisms have been investigated in terms of their single-molecule photodynamics. These systems consist of protein assemblies in which various chromophores are electronically coupled so that the summed absorption covers a wide spectral

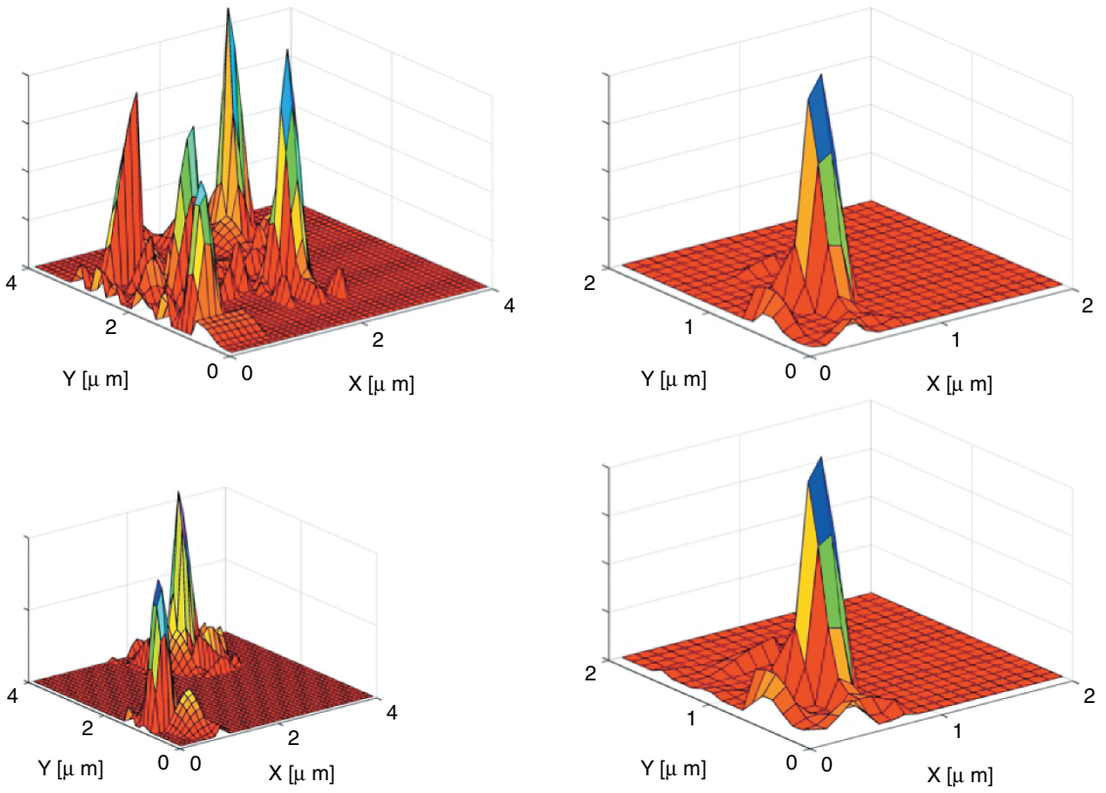

FIGURE 39.9. Fluorescence image of single proteins excited in the A band (excitation at 780 nm, emission at 440 nm) (upper left). (Right) Same field of view but on the B channel (excitation at 880 nm emission at 535 nm). (Lower panel, left) Fluorescent image in the A channel after irradiation at 710 nm (2 mW and 50 ms). (Lower panel, right) Same field of view but on the B channel and after irradiation at 720 nm (2 mW and 50 ms). Color levels of left panels are five times those in right panels. (After Chirico *et al.*, 2004.)

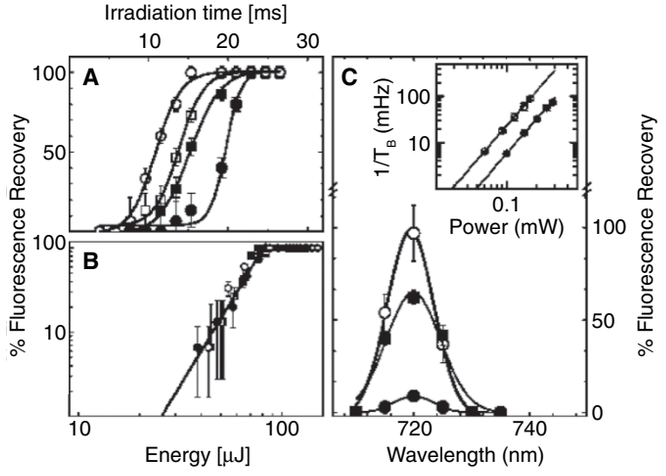

FIGURE 39.10. (A) Percentage of single-protein fluorescence recovery versus the illumination time at 720 nm for different excitation powers: 3.34 mW (*solid circles*), 4.34 mW (*solid squares*), 5 mW (*open squares*), and 5.7 mW (*open circles*). The solid lines are best-fit to a sigmoidal function. (B) Recovery efficiency with laser excitation at 720 nm versus the product of the illumination time with excitation power: 3.34 mW (*solid circles*), 4.34 mW (*solid squares*), 5 mW (*open squares*), and 5.7 mW (*open circles*). The solid line is the best-fit power law with an exponent of 3.8 ± 0.2. (C) Recovery efficiency at a fixed illumination time of 16.5 ms versus the excitation wavelength at 5.7 mW (*open circles*), 4.34 mW (*filled squares*), and 3.34 mW (*filled circles*). The error bars are the 95% statistical errors due to the number of events observed. The inset shows the photobleaching rate $1/T_b$ versus excitation power for the two states (A, *open squares*; B, *filled squares*). (After Chirico *et al.*, 2004.)

range. A subtle interplay between different electronic coupling mechanisms is responsible for an efficient transfer of the absorbed energy to the reaction center. Recently, a study of photobleaching and energy transfer in single phycoerythrocyanin (PEC) monomer has been presented by Zehetmayer and colleagues (2002). The PEC monomer contains two different chromophores, phycoviolobilin (PVB) and phycocyanobilin (PCB) (Zhao and Scheer, 1995). Zehetmayer and co-workers (2002) recorded single-molecule images of phycoerythrocyanin monomers. Their photobleaching behavior was studied by simultaneously exciting at two wavelengths according to a method earlier applied on the E222Q GFP mutant by the same group (Jung *et al.*, 2001). The PVB chromophore was found to be responsible for the photobleaching of PEC. On the other hand, it was possible to ascertain that the 15E form of PVB corresponds to one of the short-lived dark states of PEC. This form does not induce real photobleaching but simply reduces the average fluorescence emission, as is the case in most of the GFP mutants. The difficulty in discriminating between nonreversible photobleaching and long-term blinking therefore seems to be ubiquitous in single-protein photophysics.

CONCLUSION

It is not easy to indicate conclusions that define an optimal strategy for either reducing or exploiting the photobleaching process. We hope that the facts reported in this chapter could be useful to design new experiments or to revise old ones with the aim of a better understanding of the delicate, intricate, and complex structure–function

relationship that is the basis of our job as microscopists or, more generally, as biophysicists. We decided to attack photobleaching by considering both its imaging and its single-molecule aspects because we think that feedback between these related aspects of the problem can greatly improve our knowledge of photobleaching.

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